Evaluation of Non-Lethal Effects of N_2 Bubbles on Marine Mammal Health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury

Laura Thompson & Tracy Romano
Mystic Aquarium
55 Coogan Blvd Mystic CT 06355
phone: (860) 572 5955 x155 email: lthompson@mysticaquarium.org
email: tromano@mysticaquarium.org

Award Number: N000141512203

LONG-TERM GOALS

The major purpose of this project is to investigate the potential non-lethal effects of gas bubbles on immune function in marine mammals and to address the hypothesis that a less reactive immune system serves a protective role against development of decompression sickness (DCS).

OBJECTIVES

1) Measure phagocytosis and respiratory burst, as well as granulocyte activation, in marine mammal blood samples following in vitro exposure to N_2 bubbles.
2) Monitor changes in complement components (complement activation) in marine mammal blood samples following in vitro exposures to N_2 bubbles.
3) Evaluate the role of exercise modulation on the response of immune cells and complement activation to diving and in vitro exposures to N_2 bubbles.
4) Validate the presence of complement proteins in blow for potential use in monitoring immune status in belugas.

APPROACH

Blood samples will be obtained from resident beluga whales (n=3), as well as from stranded harbor seals, harp seals and grey seals (objectives 1 and 2) which are admitted to the Animal Rescue Program at Mystic Aquarium between 2015 and 2016. Blood samples will also be collected from wild belugas belonging to the Bristol Bay and Chukchi Sea, Alaska populations during live capture/release health assessments or subsistence hunts in 2016 (target n=10 per season/per location). Initial processing will occur in the field and samples will be shipped back in liquid nitrogen for analysis of hormones and complement. For objective 4, blow samples will be obtained from belugas (n=3) at the Mystic Aquarium, as well as animals from the Bristol Bay and Point Lay, AK populations.

Plasma stress hormones will be monitored in all samples to control for confounding effects of changing physiological status. Cortisol will be measured using a commercial enzyme immunoassay (EIA) and plasma catecholamines will be measured using a Waters (Milford, MA) High Performance Liquid Chromotography system(1515 isocratic pump, 717 autosampler) with Electrochemical Detection (2465 electrochemical detector).

In order to measure immune responses to bubbles, nitrogen bubbles will be introduced to blood samples *in vitro* using a micro-flowmeter (Bergh *et al.,* 1993). Various flow rates or bubble sizes will be targeted as feasible in order to evaluate whether there is a threshold which determines symptomatic vs. asymptomatic immune responses.

Method for Objective 1: Measure phagocytosis and respiratory burst in marine mammal blood samples following in vitro exposure to N2 bubbles. Phagocytosis and respiratory burst will be measured in granulocyte and monocyte populations using the protocol detailed in Spoon and Romano (2012) which was developed for use with marine mammal samples with prior ONR support. Briefly, propidium iodide labeled *Staphylococcus aureus* and dichlorofluorescein diacetate (DCFDA) are added to whole blood samples which have also been exposed to N2 bubbles. Non-exposed samples will be used as control measures. Following predetermined incubations of 10 and 75 minutes N-ethylmaleimide (NEM) will be added to stop cellular activity and red cells will be lysed. Flow cytometry will then be used to monitor bacterial uptake (phagocytosis) and DCF fluorescence in response to production of oxygen radicals (respiratory burst). In addition, a commercially available mouse-anti-canine antibody for CD11b (AbDserotec), shown to cross-react with whale and seal samples (Thompson, 2014), will be used to monitor expression of this protein on granulocytes as a marker of activation. In addition, the expression of CD18 will be investigated as a marker of activation. Following exposures to N2 bubbles, whole blood will be incubated with CD11b or CD18 antibody for 30 minutes, washed, and incubated with a FITC conjugated secondary antibody for an additional 30 minutes. Red cells will be lysed and florescence, corresponding with expression, will be measured using flow cytometry. Measurements of cell function will be compared with and without bubble exposures, as well as between different exposures.

Method for Specific Aim 2: Monitor changes in complement components (complement activation) in marine mammal blood samples following in vitro exposures to N2 bubbles. Several methods exist for measurement of complement proteins to evaluate complement activation. Commercial EIA's for specific complement proteins (e.g. C5A), as well as a complement activation Elisa which measures total activity of the alternative pathway, will be purchased and validated for use with marine mammal samples.

Method for Specific Aim 3: Evaluate the role of exercise modulation on the response of immune cells and complement to in vitro exposures to N2 bubbles. Belugas at Mystic Aquarium will be trained to perform vigorous underwater swimming behaviors prior to blood sampling in order to investigate the effects of exercise on immune function and complement activation (measured as per specific aims 1 and 2). Blood sampling at various intervals following activity (e.g. immediate vs. 1 hour vs. 24 hours) will be targeted. Results will be compared with those from blood samples drawn following stationary training sessions.

Method for Specific Aim 4: To validate the presence of complement proteins in blow for potential use in monitoring immune status in belugas. Blow samples will be collected from belugas to determine the presence of complement. Previous work performed under ONR funding (Award No # N00014-11-1-

0437) determined blow sampling to be a useful non-invasive tool for monitoring cortisol in belugas (Thompson et al., 2014), and this work would aim to evaluate the additional potential for blow samples to provide a non-invasive means of evaluating risk or susceptibility to dive related injury in free-ranging cetacean populations

WORK COMPLETED

Due to a delay in the distribution of funds, the start of this project did not commence until late in the fiscal year. Unforeseen circumstances resulted in cancellation of field sampling for the summer of 2015 in Bristol Bay. In addition, whales were only seen once during the field season in Point Lay without opportunties for sampling. However, serum, plasma, buffy coat and blow samples have been collected monthly from Aquarium whales for validation purposes as well as to initiate objectives 2 and 4. Training of dive behaviors has begun for objective 3. Equipment for the introduction of N2 gas bubbles to samples has been set up and the flow of gas through the microflowmeter has been tested successful. This setup will be tested with blood samples in early October, and the protocol for these experiments is expected to be finalized in mid-late October. Validation of complement EIA's for C5A and AH50 (total activey of the alternative pathway) has been initiated. Cross reactivity has been tested for plasma and serum samples from belugas, harbor seals, harp seals and grey seals. Blow samples from belugas have also been tested for cross reactivity for C5A. In addition, *in vitro* manipulation of complement activity in plasma and serum has been tested using zymosan (Sigma Aldrich) which is a known activator of complement. Validation of the EIA for complement Factor B will occur in early October. Measurement of complement activity in experimental samples is expected to begin in late October or early November, following finalization of the protocol for nitrogen bubble exposures. Experimental data collection for objectives 1,2 and 4 is expected to be underway by November 2015. Data collection for objective 3 will occur during project year 2.

RESULTS

EIA kits for seal complement factor C5A and AH50 show cross reactivity with samples from beluga, as well as harbor seals, harp seals and grey seals. A 30 minute incubation with zymosan (100 mg/ml) produces an expected increase in AH50 in both serum and NaHep plasma from all three pinniped species tested (Figure 1). This increase was not observed in belugas, but may be due to species specific sensitivities. Plasma from harbor seals and belugas, collected in EDTA vacutainers displayed no change in AH50 in the presence of zymosan (Figure 2). This is as expected as EDTA is known to block complement activity.

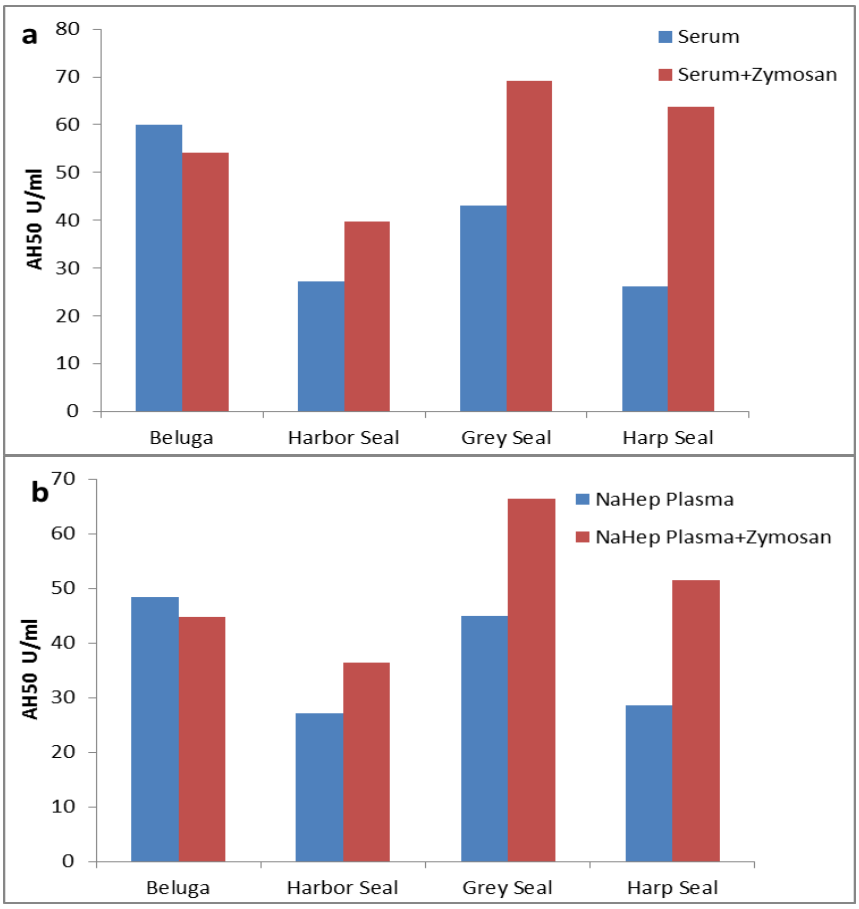

Figure 1: Zymosan stimulation of the alternate complement pathway in a) serum and b) NaHep plasma samples from a beluga, harbor seal, grey seal, and harp seal.

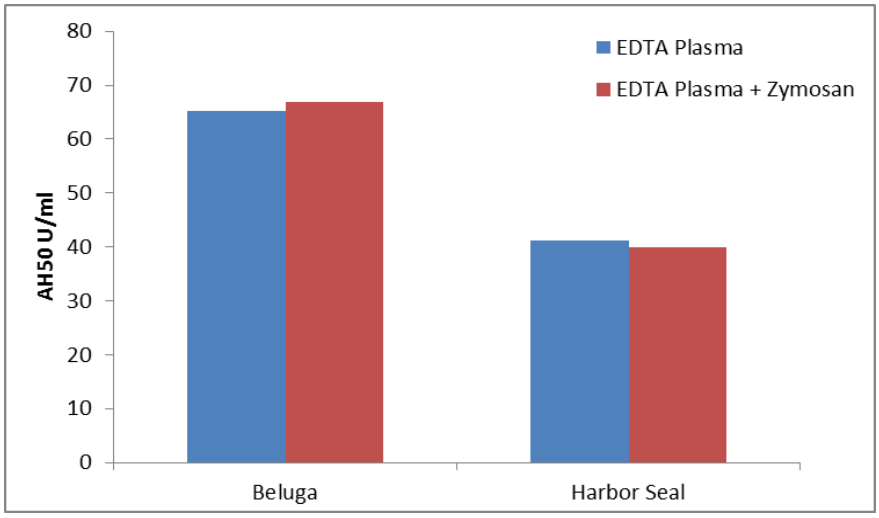

Figure 2: Zymosan stimulation of the alternate complement pathway in EDTA plasma samples from a beluga and harbor seal.

Complement component C5 has been detected in serum and NaHep plasma in belugas, harbor seals and grey seals. Values measured in NaHep plasma appear to be greater than those obtained from serum, which is not surprising as *in vitro* activation of complement due to sampling methods or storage has been reported. Interestingly, C5 was also detectable in a beluga blow sample tested, at slightly lower values than in serum (Figure 3).

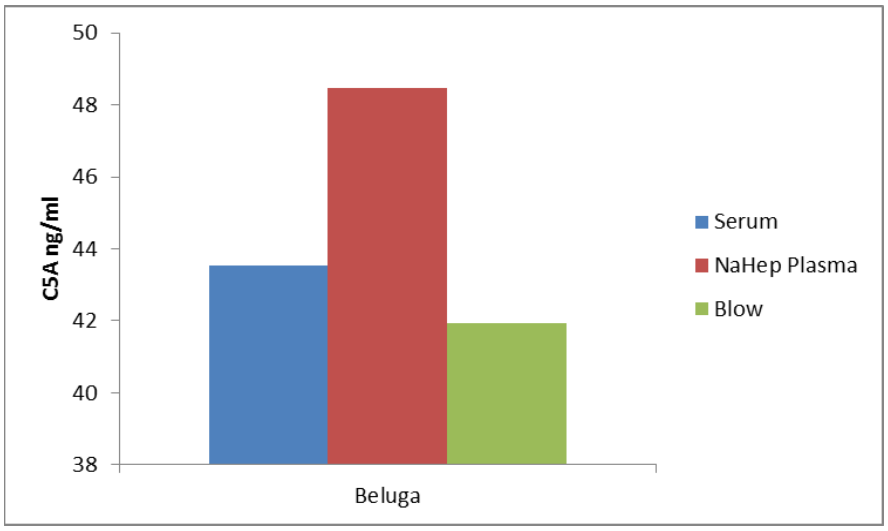

Figure 3: Complement component 5A levels in beluga serum, NaHep plasma and blow samples.

IMPACT/APPLICATIONS

The proposed work builds on previous ONR funded research exploring the relationship between marine mammal health, dive physiology and stressors. This study has relevance to the ONR Marine Mammals and Biology Program's research interest in the effects of sound on marine life, as noise or sound exposure can potentially alter dive patterns/behavior. It will also increase understanding of marine mammal dive physiology and how these animals avoid developing dive related pathologies. Previously funded research (award N00014-13-1-0768) was the first to investigate immune function in marine mammals within the context of diving and the findings suggest that there is a dynamic relationship between dive behavior, immune function and health. This research will provide additional knowledge as to function of marine mammal immune responses during diving and development of dive related disease. This work has future implications for understanding the occurrence of dive related pathologies in marine mammals and interpreting integrated effects of human activities on marine mammal health.

RELATED PROJECTS

ONR Award N00014-13-1-0768
Evaluating the Effects of Stressors on Immune Function During Simulated Dives in Marine Mammals

ONR Award N00014-11-1-0437
Investigation of the Physiological Responses of Belugas to "Stressors" to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health

REFERENCES

Bergh, K., Hjelde, A., Iversen, O-J., Brubakk, A.O. 1993. Variability over time of complement activation induced by air bubbles in human and rabbit sera. *J Appl Physiol* 74:1811-1815.

Spoon, T. and Romano, T.A. 2012 Neuroimmunological Response of Beluga Whales (*Delphinapterus leucas)* to Translocation and Social Change. *Brain Behav Immunol* 26:122-131.

Thompson, L.A. 2014. The Combined Influence of Diving Physiology and Stressors on Immune Cell Functions in a Deep Diving Monodontid and Three Shallow Diving Phocid Species. *PhD Thesis,* University of Connecticut, Avery Point.

Thompson, L.A., Spoon, T.R., Goertz, C.E.C., Hobbs, R.C., Romano, T.A. 2014. Blow Collection as a Non-Invasive Method for Measuring Cortisol in the Beluga (*Delphinapterus leucas*). *PLoS One.* 9:e114062.